Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 6528–6532, September 1988
Medical Sciences

Amphiregulin: A bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7

(cancer/differentiation/cytokine/growth factor/growth inhibitor)

MOHAMMED SHOYAB, VICKI L. MCDONALD, J. GARRETT BRADLEY, AND GEORGE J. TODARO

Oncogen, 3005 First Avenue, Seattle, WA 98121

Contributed by George J. Todaro, May 9, 1988

ABSTRACT **A glycoprotein, termed amphiregulin (AR), inhibits growth of several human carcinoma cells in culture and stimulates proliferation of human fibroblasts and certain other tumor cells. It has been purified to apparent homogeneity from serum-free conditioned medium of MCF-7 human breast carcinoma cells that had been treated with phorbol 12-myristate 13-acetate. AR is a single-chain extremely hydrophilic glycoprotein containing cysteines in disulfide linkage(s) that are essential for biological activity; it is stable between pH 2 and pH 12 and after heating for 30 min at 56°C but unstable at 100°C. The apparent molecular weights of AR and N-Glycanase-treated AR are 14,000 and 15,000, respectively, as assessed by gel chromatography, and ≈22,500 and ≈14,000, respectively, as determined by polyacrylamide gel electrophoresis. Treatment of AR with N-Glycanase, O-Glycanase, or neuraminidase does not affect its activity. The pI of AR is ≈7.8. The amino-terminal amino acid sequence of AR has been determined, and no significant sequence homology between AR and other proteins was found. The molecule thus appears to be a distinct growth regulatory protein.**

Cellular growth and differentiation appear to be initiated, promoted, maintained, and regulated by a multiplicity of stimulatory, inhibitory, and synergistic factors and hormones. The alteration and/or breakdown of the cellular homeostasis mechanism is a fundamental cause of growth-related diseases including neoplasia (1–11). Physiological regulators of cell growth and differentiation, such as peptide growth factors (2, 4, 10, 11), hematopoietic regulatory proteins (5, 12–14), tumor necrosis factor types α and β (8, 9), interferons (15), tumor inhibitory factor types 1 and 2 (16, 17), oncostatin M (18), leukoregulin (19), and other less well-defined factors (20–22) can inhibit the proliferation of certain tumor cells, alter the tumor-cell phenotypes, and induce or modulate cell differentiation *in vitro* and *in vivo*. Furthermore, most of these regulatory factors elicit multiple biological effects that depend on the target cells and assay conditions (23). Biologically active phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), are potent tumor-promoters *in vivo*, and they elicit and modulate a wide variety of biological and biochemical responses *in vivo* as well as *in vitro* (24–26). It has been known for some time that PMA inhibits the growth of human breast adenocarcinoma cell line MCF-7 (27). In addition, PMA also alters the morphology of MCF-7 cells and PMA-treated cells exhibit the morphological characteristics of secretory cells (27, 28). We have thus undertaken studies aimed at isolating and characterizing growth modulatory factors produced by MCF-7 cells in response to PMA treatment. Here we report that PMA-treated MCF-7 cells release a glycoprotein that inhibits the growth of several human carcinoma cell lines but augments the growth of normal human fibroblasts and some other cell lines.

MATERIALS AND METHODS

Cell Lines. All cell lines used were obtained from the American Type Culture Collection or from our own cell bank. Human forearm fibroblast lines (SS and WHG) were provided by M. A. Bean (Virginia Mason Research Center, Seattle). Cells were maintained in standard medium [Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, or 50% (vol/vol) DMEM and 50% (vol/vol) Iscove's modified Dulbecco's medium (IMDM)] supplemented with 5–15% (vol/vol) heat-inactivated fetal bovine serum, L-glutamine, penicillin (60.6 μg/ml), and streptomycin (100 μg/ml).

Growth Modulatory Assay with ^{125}I-Labeled Deoxyuridine Incorporation into DNA. The assays were performed in 96-well flat-bottomed plates (Falcon, catalog number 3072). Human epidermoid carcinoma of the vulva cells (A431) were used as test cells for growth inhibitory activity (GIA) and human forearm fibroblasts (SS) were used as indicator cells for growth stimulatory activity. A total of 3.5×10^4 cells in 50 μl of DMEM, supplemented with 5% (vol/vol) heat-inactivated fetal bovine serum, penicillin (60.6 μg/ml), streptomycin (100 μg/ml), and glutamine (test medium), was placed in all wells except peripheral wells. Three hours later, 50 μl of the test sample in test medium was added to each well; control wells received only 50 μl of test medium. Three wells were used for each concentration of test sample. Plates were incubated at 37°C for 2–3 days. After this, 100 μl of solution of ^{125}I-labeled deoxyuridine (Amersham) [4 Ci/mg; 0.5 Ci/ml (2 μl/ml in test medium); 1 Ci = 37 GBq] was added to each well and plates were incubated at 37°C. After 4–6 hr, samples were processed as described by Iwata *et al.* (16).

Production of Crude Amphiregulin (AR). MCF-7 cells were cultured in T150 Corning tissue culture flasks in a total volume of 25 ml of 50% IMDM/50% DMEM containing insulin (0.6 μg/ml) and 15% (vol/vol) heat-inactivated fetal bovine serum (complete medium). On day 6, all medium was removed and 20 ml of fresh MCF-7 complete medium containing PMA (100 ng/ml) was added to each flask. Forty-eight hours later, the medium was removed and each flask was rinsed with 15 ml of 50% IMDM/50% DMEM (serum-free medium) and 25 ml of fresh serum-free medium was added to each flask and incubated at 37°C with an atmosphere of 5% CO_2/95% air. Four days later, the conditioned serum-free medium was collected, centrifuged to remove debris, and stored at −20°C. Flasks were again fed with 25 ml of serum-free medium and conditioned serum-free

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; GIA, growth inhibitory activity; F_3CCOOH, trifluoroacetic acid; AR, amphiregulin; rp, reverse phase; SPE-AR, S-pyridylethylated AR.

medium was collected every third or fourth day. An aliquot of the conditioned medium from each collection was assayed for GIA. Usually, three to four rounds of conditioned medium were collected from each batch of PMA-treated MCF-7 cells.

About 4.5 liters of conditioned medium was thawed and centrifuged at 4°C for 15 min at 3500 rpm in a TYJS 4.2 rotor. The supernatant was concentrated in an Amicon 2-liter concentrator with a YM10 membrane (Amicon) at 4°C. When the volume of retenate became about 200 ml, 1 liter of cold Milli-Q-purified water was added and the mixture was re-concentrated to 200 ml.

The concentrate was removed and transferred to a pre-cooled 250-ml Corning centrifuge bottle. Concentrated acetic acid was slowly added with stirring to a final concentration of 1 M acetic acid. The mixture was allowed to stand for 1 hr at 4°C and centrifuged for 20 min at 40,000 × *g* at 4°C. The supernatant was removed and dialyzed in Spectrapore dialysis tubing (number 3, molecular weight cutoff ≈3000) against 17 liters of 0.1 M acetic acid. The dialysis buffer was changed three times over a 2-day period. The retenate was lyophilized. The dry material was removed, pooled, weighed, and stored at −20°C. We call this material crude powder.

Purification of AR. Crude powder (950 mg from ≈9 liters of serum-free conditioned medium) was suspended in 300 ml of 0.1% trifluoroacetic acid (F_3CCOOH) and centrifuged for 20 min at 7000 × *g*. The supernatant was carefully removed and applied on a column of preparative C_{18} (2.54 cm × 27 cm; 55–105 μm; Waters Division of Millipore) equilibrated with 0.1% F_3CCOOH in water. The flow rate was 4 ml/min and the chromatography was carried out at room temperature. The column was washed with 650 ml of 0.1% F_3CCOOH in water. The flow through and wash were collected together. About 77% of total GIA activity appeared in the flow through and wash.

The flow-through and wash fraction were injected isocratically onto a preparative Partisil 10 ODS-3 column (10 μm, 2.2 × 50 cm, Whatman) attached to a HPLC system (Waters Division of Millipore). The flow rate was set at 4 ml/min. Once the sample had passed on to the column, the column was washed with 250 ml of 0.1% F_3CCOOH in water. The linear gradient was used between the primary solvent, 0.1% F_3CCOOH in water, and the secondary solvent MeCN containing 0.1% F_3CCOOH. The gradient conditions were as follows: 0–15% MeCN in 10 min, 15–15% MeCN in 30 min, 15–25% MeCN in 150 min, 25–65% MeCN in 100 min, and 65–100% MeCN in 10 min. Fractions of 14 ml were collected and aliquots of each fraction were assayed for GIA. Two broad peaks of activity were seen (Fig. 1*A*). The early peak (eluting between 20% and 23% MeCN) was further purified and characterized.

Fractions 47–62 were pooled and to that was added 224 ml of 0.1% F_3CCOOH in water. The mixture was isocratically injected onto a semipreparative μ-Bondapak-C_{18} column (7.8 × 300 mm, Waters Division of Millipore). The linear gradient conditions were 0–17% acetonitrile in 10 min, 17–17% acetonitrile in 30 min, 17–25% acetonitrile in 320 min, and 25–100% acetonitrile in 40 min. The flow rate was 1 ml/min during the gradient and 4-ml fractions were collected. Two major peaks of activity were observed, eluting at acetonitrile concentrations of ≈20% and ≈21%, respectively (Fig. 1*B*).

Fractions 49–53 were pooled and 20 ml of 0.1% F_3CCOOH was then added to the pooled fraction. The mixture was applied onto an analytical μ-Bondapak C_{18} column (3.9 × 300 mm, Waters Division of Millipore). The gradient conditions were 0–18% acetonitrile in 10 min, 18–18% acetonitrile in 30 min, 18–25% acetonitrile in 280 min, and 25–100% acetoni-

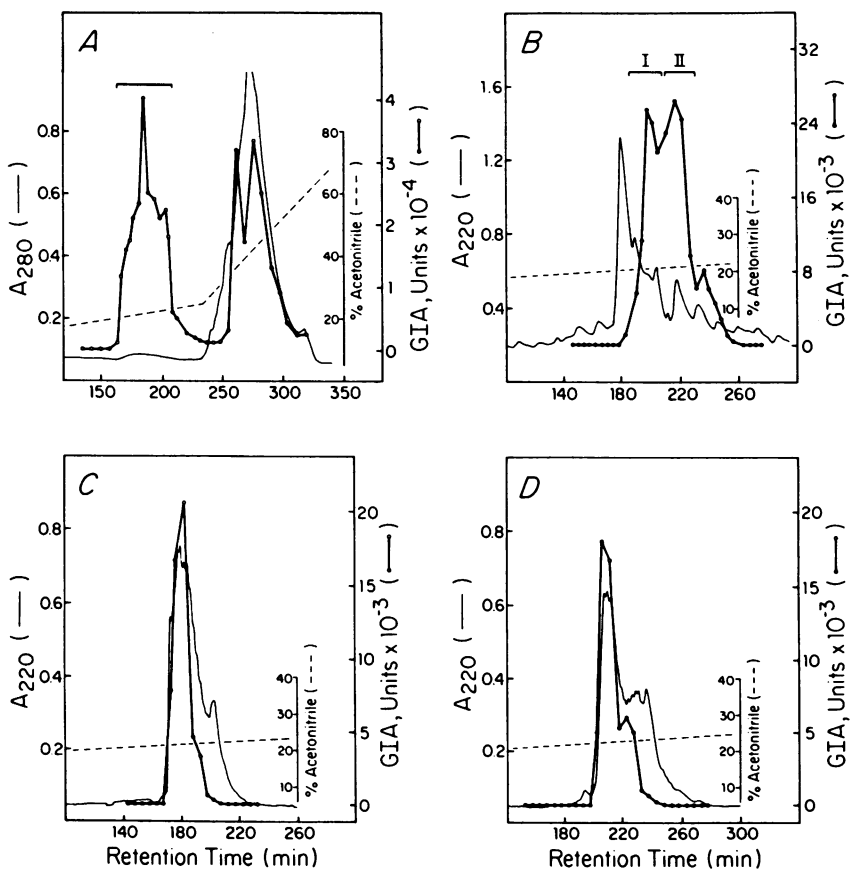

FIG. 1. Purification of AR by reverse-phase (rp) HPLC. (*A*) Preparative rpHPLC of break through and wash. (*B*) Semipreparative rpHPLC of pooled fraction 47–62 from *A*. (*C*) Analytical rpHPLC of pool I from *B*. (D) Analytical rpHPLC of pool II from *B*.

trile in 20 min. The flow rate was 0.4 ml/min and 2-ml fractions were collected. Most of the activity emerged from the column at ≈21.5% acetonitrile concentration (Fig. 1*C*). Fractions 54–59 (Fig. 1*B*) were pooled and chromatographed exactly as described above in Fig. 1*C*. Most of the activity was eluted from the column at an acetonitrile concentration of ≈22.2% (Fig. 1*D*).

Fractions 35–38 (Fig. 1*C*) were individually concentrated to ≈70 μl to which was added an equal volume of acetonitrile containing 0.1% F_3CCOOH. This 140-μl sample was injected onto two Bio-Sil TSK-250 columns (7.5 × 300 mm each, Bio-Rad) arranged in tandem. The elution was performed isocratically with a mobile phase of 50% (vol/vol) MeCN in H_2O with 0.1% F_3CCOOH at room temperature. The flow rate was 0.4 ml/min, 0.4-ml fractions were collected, and aliquots were assayed for GIA. Chromatographic profiles of fractions 35, 36, and 37 (Fig. 1*C*) are shown in Fig. 2 *A–C*, respectively.

Fractions 41 and 42 (Fig. 1*D*) were pooled, concentrated to 70 μl, and then subjected to gel chromatography as described above. The chromatographic profile is given in Fig. 2*D*.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Proteins were analyzed on $NaDodSO_4$/polyacrylamide slab gels [normal or mini Bio-Rad system by the method of Laemmli (29)]. Proteins were detected by silver staining (30).

Amino Acid Sequence Determination. For amino-terminal sequence analysis, AR was reduced with 2-mercaptoethanol and alkylated with 4-vinylpyridine. S-pyridylethylated AR (SPE-AR) was purified by rpHPLC with a μBondapak C_{18} column. Peptide sequences were determined with an Applied Biosystems (Foster City, CA) model 475A gas-phase sequencer by the method of Hewick *et al.* (31). Three precycles of Edman degradation were performed prior to sample application. Identification of phenylthiohydantoin amino acid derivatives was carried out, on-line, on a model 120A phenylthiohydantoin analyzer (Applied Biosystems) by the method of Hunkapiller and Hood (32).

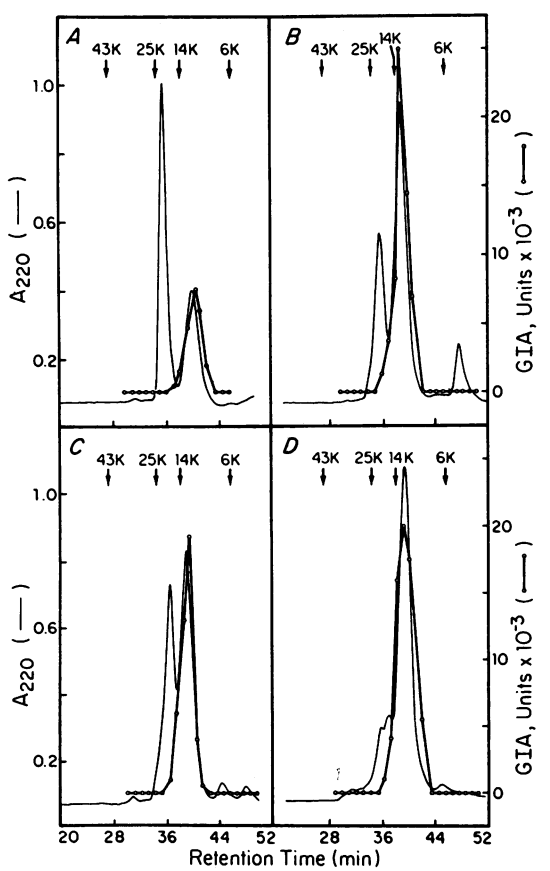

FIG. 2. Gel chromatographies of fractions from Fig. 1 *C* and *D* on Bio-Sil TSK 250 columns. (*A*) HPLC of concentrated fraction 35 (Fig. 1*C*). (*B*) HPLC of concentrated fraction 36 (Fig. 1*C*). (*C*) HPLC of concentrated fraction 37 (Fig. 1*C*). (*D*) HPLC of concentrated fractions 41 and 42 (Fig. 1*D*).

RESULTS AND DISCUSSION

Production of AR by PMA-Treated MCF-7 Cells. MCF-7 cells grown on a plastic substratum exhibit epitheloid features: cells are small and polygonal in shape. PMA induces dramatic morphological changes in a dose-dependent manner. After PMA treatment, MCF-7 cells lose their well-defined morphology, become rounded, and spread out. PMA elicits a dose-dependent reduction in cell number and an increase in cell volume compared to untreated cells.

Serum-free conditioned medium from MCF-7 cells did not contain any detectable growth inhibitory activity for A431 cells. However, serum-free media collected from MCF-7 cells treated with PMA for 2–3 days were found to inhibit the proliferation of A431 epidermoid carcinoma cells. The optimum induction of inhibitory activity was observed at PMA concentrations between 25 and 100 ng/ml. Even after removing PMA, PMA-treated MCF-7 cells released this activity in serum-free medium for at least 8 days. Although a reduction in growth inhibitory activity was noticed with time after PMA removal, these results indicate that AR is either induced or increased in expression in MCF-7 cells that have been treated with PMA.

Initial Characterization of AR. The GIA was resistant to treatment with 1 M acetic acid, 1 M ammonium hydroxide, 6 M urea, 0.01 M sodium metaperiodate, to heating at 56°C for 30 min, and to treatments with neuraminidase, N-Glycanase, O-Glycanase, lipase, or phospholipase A2, C, or D. However, activity was sensitive to heating at 100°C for 10 min, to reduction, to reduction and 4-vinylpyridine treatment, and to digestion with proteinases such as trypsin, endoproteinase Lys-C, endoproteinase Arg-N, and endoproteinase Glu-C (V-8). These results suggest that AR is a protein containing cysteines in disulfide linkage(s) that are essential for its biological activity. This protein may contain oligosaccharides and/or lipid moieties that are not obligatory for the biological activity.

Purification of AR and Certain Physical Properties. A summary of AR purification is presented in Table 1. A 1842-fold purification with 5.1% yield has been achieved for

Table 1. Summary of purification of AR (GIA)

Fraction	Protein, μg	GIA units,* no. × 10^{-3}	Specific activity, units/mg, no. × 10^{-3}	Yield, %
Crude	916,600	1363†	1.49	100
Prep. C_{18}	532,000	1052†	1.98	77.2
Prep. ODS	2,281	286	125	21.0
Semiprep.				
C_{18}-I	339	97	286	7.1
C_{18}-II	235	92	391	6.7
Anal. C_{18}-I	242	50	206	3.7
Anal. C_{18}-II	173	46	265	3.4
TSK 250-I	25.5	70	2740	5.1
-I-A	6.6	15	2270	1.1
-I-B	11.4	33	2890	2.4
-I-C	7.5	22	2930	1.6
TSK 250-II	6.8	23	3380	1.7

Prep., preparatory; anal., analytical.
*One unit of GIA is the amount of factor required to inhibit ^{125}I-labeled deoxyuridine incorporation into A431 cells by 50%.
†Other growth inhibitory activities are present in these fractions. These values include all activities.

TSK 250-I fraction and a 2270-fold purification with 1.7% yield has been obtained for TSK 250-II fraction. The method is reproducible. The specific activity of purified AR was 2.7–3.4 × 10^6 units/mg of protein. The molecular weight of AR and SPE-AR, determined by gel chromatography on a TSK-250 column, was ≈14,000 and ≈17,000, respectively.

Fig. 3 shows an analysis of AR, N-Glycanase-treated AR, SPE-AR, and N-Glycanase-treated SPE-AR in a 15% polyacrylamide gel under reducing conditions. AR and SPE-AR migrated in the gel as a broad, diffused single band with a median relative molecular weight of 22,500 and a range of 20,000–25,000 (lanes 1 and 3). The treatment of AR and SPE-AR with N-Glycanase resulted in the faster migration of these proteins. N-Glycanase-treated AR and N-Glycanase-treated SPE-AR migrated as single bands with median molecular weights of 14,000 and 14,500, respectively (lanes 2 and 4). Similar results were observed when proteins were electrophoresed in a 15% gel under nonreducing conditions (data not shown). The treatment of AR with neuraminidase, O-Glycanase, or neuraminidase plus O-Glycanase did not alter its electrophoretic mobility under either reducing or nonreducing conditions. Thus, AR is a single-chain glycoprotein containing an N-linked oligosaccharide chain(s) that is not required for the GIA of AR *in vitro*.

Isoelectrofocusing analysis of AR and N-Glycanase-treated AR was performed. AR focused as a single broad band with a pI of between 7.7 and 8.0, and N-Glycanase-treated AR focused as a single band with pI of ≈8.1. Thus AR is a basic single-chain glycoprotein containing N-linked oligosaccharides.

Amino-Terminal Amino Acid Sequence of AR. Automated Edman degradation of reduced SPE-AR was performed with 385 pmol of protein. The partial amino-terminal acid sequences of truncated AR and of a larger protein containing six additional amino acids at amino-terminal end are as follows

Truncated AR
^{1}V V K P P Q D K T ^{10}E S E N T S D K P K ^{20}R K K K G G

Larger AR
^{1}S V R V E Q V V K ^{10}P P Q D K T E S E N ^{20}T S

The amount of larger AR was found to be ≈20% that of truncated form. Unambiguous identification of phenylthiohydantoin amino acid derivatives was possible up to residue

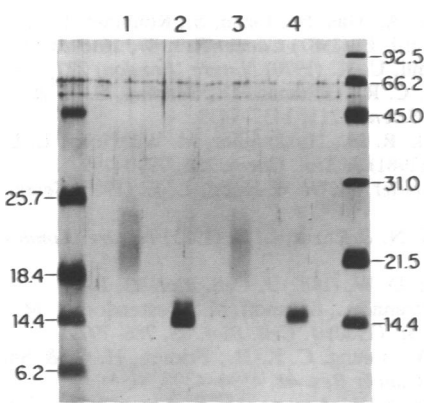

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoretic analysis of AR. A 15% $NaDodSO_4$/polyacrylamide gel (0.75 mm × 18 cm × 15 cm, Bio-Rad) with a discontinuous buffer system was electrophoresed at a constant current of 30 mA as described. The markers were phosphorylase B, bovine serum albumin, ovalbumin, carbonic anydrase, chymotrypsinogen A, soybean trypsin inhibitor, β-lactoglobulin, lysozyme, aprotonin, and insulin subunit. Lanes: 1, AR; 2, N-Glycanase-treated AR; 3, SPE-AR; 4, N-Glycanase-treated SPE-AR. Molecular weights (×10^{-3}) are shown.

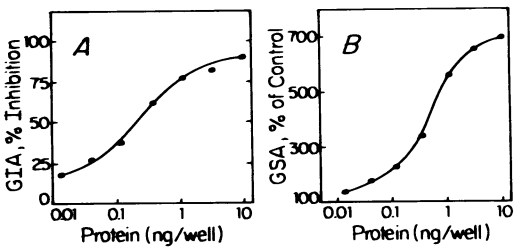

FIG. 4. (*A*) Dose–response curve of AR on the inhibition of ^{125}I-labeled deoxyuridine incorporation into DNA of A431 cells. (*B*) Effect of AR on the stimulation of ^{125}I-labeled deoxyuridine incorporation into DNA of human forearm fibroblasts (SS). GSA, growth-stimulatory activity.

25 for truncated AR and up to residue 21 for the larger form of the protein. There is one potential *N*-glycosylation site (underlined) in the above sequences. It should be noted that AR contains the unusual sequence Lys-Arg-Lys-Lys-Lys at positions 19–23 in the truncated form.

The partial amino acid sequence of AR was compared with all proteins in the National Biomedical Research Foundation data base,* Genetic Sequence Database,† and European Molecular Biology Laboratory DNA Sequence Library.‡ These searches did not reveal any substantial sequence similarity between the amino-terminal amino acid sequence of AR and any other known sequence.

Biological Properties of AR. The inhibition of ^{125}I-labeled deoxyuridine incorporation into DNA of A431 cells by various concentrations of the purified AR is given in Fig. 4*A*. A 50% inhibition of DNA synthesis was observed at ≈0.1 ng per well (0.13 nM AR). However, it should be noted that GIA of the AR depends on experimental conditions such as number of cells per well (cell density), time of factor application, duration of treatment, serum concentration, and other variables.

The stimulation of ^{125}I-labeled deoxyuridine incorporation into DNA of human forearm fibroblasts (SS) by various concentrations of the purified AR is shown in Fig. 4*B*. A 2-fold stimulation of ^{125}I-labeled deoxyuridine incorporation was seen at ≈0.07 ng/ml (0.09 nM AR). The maximum response was an ≈6-fold stimulation of ^{125}I-labeled deoxyuridine incorporation into these fibroblasts. Thus, AR acts as a growth factor for human fibroblasts even in 5% (vol/vol) fetal bovine serum.

The effect of AR on the incorporation of ^{125}I-labeled deoxyuridine into the DNA of various tumor and nontumor human cell lines and some nonhuman cell lines was investigated. The data are presented in Table 2. AR inhibited the growth of various clones of A431 cells, human breast carcinoma cells HTB 132, human ovary teratocarcinoma cells HTB 1575, human epidermal carcinoma of cervix cells CRL 155, human papillary adenoma of ovary cells HTB 75, human neuroblastoma cells HTB 10, and human breast carcinoma cells HTB 26. It did not exhibit any significant effect on various other cells such as human melanoma A375, human adenocarcinoma of breast ZR, human adenocarcinoma of breast MCF-7, human adenocarcinoma of lung A549, human carcinoma of colon H3347, human lymphoblastoid (T cells) CEM, human Epstein–Barr virus-transformed B cells, human epidermal carcinoma of larynx Hep 2, bovine fetal heart endothelial cell CRL 1395, murine BALB/3T3, and mink lung

*Protein Identification Resource (1988) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 15.
†EMBL/GenBank Genetic Sequence Database (1988) GenBank (IntelliGenetics, Mountain View, CA), Tape Release 54.
‡EMBL/GenBank Genetic Sequence Database (1987) EMBL Nucleotide Sequence Data Library (Eur. Mol. Biol. Lab., Heidelberg), Tape Release 13.

Table 2. Effects of AR on the proliferation of cells

Indicator cell	Activity, units
GIA*	
Human epidermal carcinoma of vulva A431	125
Human adenocarcinoma of breast HTB 132	243
Human epidermoid carcinoma of cervix CRL 1550	28
Human papilary adenoma of ovary HTB 75	19
Human teratocarcinoma of ovary HTB 1572	55
Human adenocarcinoma of breast HTB 26	5
Human neuroblastoma HTB 10	12
GSA†	
Human pituitary tumor CRL 7386	62
Human adenocarcinoma of ovary HTB 77	25
Human forearm fibroblasts (SS)	225
Human forearm fibroblasts (WHG)	70
Simian African green monkey kidney BSC-1	65
Rat kidney NRK-SA6	45

AR [≈125 units (GIA on A431 cells)] was suspended in 250 μl of test medium, serially diluted 1:5 with test medium. Six dilutions were used for each cell. The fractions were assayed for growth modulatory activity and the GIA and growth-stimulatory activity (GSA) units were calculated.

*One GIA unit was defined as the amount of factor needed to inhibit ^{125}I-labeled deoxyuridine incorporation into cells by 50%.

†One GSA unit was defined as the amount of factor required to increase ^{125}I-labeled deoxyuridine incorporation into cells by 100%.

CCL 64. AR stimulated the incorporation of ^{125}I-labeled deoxyuridine into several human fibroblast cell lines, human pituitary tumor cells CRL 7386, human ovarian carcinoma cells HTB77, African green monkey kidney cells BSC-1, and rat kidney cells NRK. It should be noted that epidermal growth factor is known to inhibit the proliferation of A431 human epidermoid carcinoma cells (33, 34), GH_4 rat pituitary tumor cells (35), and certain human breast cancer cells (36) while stimulating the proliferation of a number of cells in culture.

Epidermal growth factor or transforming growth factor type α induce anchorage-independent growth of NRK cells in the presence of transforming growth factor type β (37). We found that epidermal growth factor induced anchorage-independent growth of NRK cells in a dose-dependent manner in the presence of transforming growth factor type β, whereas AR was found to be a very weak inducer of colony formation of NRK cells in soft agar.

The biological, biochemical, and physicochemical properties of AR described, as well as its amino acid composition and partial amino acid sequence, therefore, would strongly suggest that AR is a cell growth regulatory peptide and is not identical to any described growth inhibitory or stimulatory molecules. AR might work as an anticancer agent at least for some tumors, as it inhibits the growth of a number of tumor cells in culture while stimulating proliferation of a variety of normal cells.

AR is shed by MCF-7 cells in response to PMA, a powerful tumor-promoting agent *in vitro* as well as *in vivo*. It is possible that AR might be expressed and secreted during early development of neoplasia thus making it a potential early marker of cancer. Further studies on the cloning, structure, topology, expression, and regulation of the AR gene might shed light on physiological function of AR. Other growth modulatory peptides are multifunctional (23). We would expect the same to be the case for AR.

We thank Ms. Phyllis Yoshida for her assistance in the preparation of this manuscript.

1. Evered, D., Nugent, J. & Whelan, J., eds. (1985) *Ciba Found. Symp.* **116**.
2. Rozengurt, E. (1986) *Science* **234**, 161–166.
3. Pardee, A. B. (1987) *Cancer Res.* **47**, 1488–1491.
4. Goustin, A. S., Leof, E. B., Shipley, G. D. & Moses, H. L. (1986) *Cancer Res.* **46**, 1015–1029.
5. Sachs, L. (1986) *Sci. Am.* **254**, 40–47.
6. Nathan, C. F. (1987) *J. Clin. Invest.* **79**, 319–326.
7. Sporn, M. B. & Roberts, A. B. (1986) *J. Clin. Invest.* **78**, 329–332.
8. Old, L. J. (1987) *Nature (London)* **326**, 330–331.
9. Beutler, B. & Cerami, A. (1987) *N. Engl. J. Med.* **316**, 379–385.
10. Sporn, M. B. & Todaro, G. J. (1980) *N. Engl. J. Med.* **303**, 878–880.
11. Sporn, M. B. & Roberts, A. B. (1985) *Nature (London)* **313**, 745–747.
12. Groopman, J. E. (1987) *Cell* **50**, 5–6.
13. Marshall, C. J. (1987) *Cell* **49**, 723–725.
14. Melchers, F. & Anderson, J. (1987) *Cell* **37**, 715–720.
15. Clemens, M. J. & McNurlan, M. A. (1985) *Biochem. J.* **226**, 345–360.
16. Iwata, K. K., Fryling, C. M., Knott, W. B. & Todaro, G. J. (1985) *Cancer Res.* **45**, 2689–2694.
17. Fryling, C. M., Iwata, K. K., Johnson, P. A., Knott, W. B. & Todaro, G. J. (1985) *Cancer Res.* **45**, 2695–2699.
18. Zarling, J. M., Shoyab, M., Marquardt, H., Hanson, M. B., Lioubin, M. N. & Todaro, G. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9739–9743.
19. Ransom, J. H., Evans, C. H., McCabe, R. P., Pomato, N., Heinbaugh, J. A., Chin, M. & Hanna, M. G. (1985) *Cancer Res.* **45**, 851–862.
20. Degliantoni, G., Murphy, M., Kobayashi, M., Francis, M. K., Perussia, B. & Trinchieri, G. (1975) *J. Exp. Med.* **162**, 1512–1530.
21. Sone, S., Mutsuura, S., Ishii, K., Shirahama, T. & Tsubura, E. (1984) *Gann* **75**, 920–928.
22. Podolsky, D. K., Pleskow, D. K. & Jafari, H. (1988) *Cancer Res.* **48**, 418–424.
23. Sporn, M. B. & Roberts, A. B. (1988) *Nature (London)* **332**, 217–219.
24. Blumberg, P. M. (1981) *CRC Crit. Rev. Toxicol.* **9**,. 153–197.
25. Slaga, T. J. (1983) *Cancer Surv.* **2**, 595–612.
26. Weinstein, I. B. (1987) *J. Cell. Biochem.* **33**, 213–224.
27. Osborne, C. K., Hamilton, B., Nover, M. & Ziegler, J. (1981) *J. Clin. Invest.* **67**, 943–951.
28. Valette, A., Gas, N., Jozan, S., Roubinet, F., Dupont, M. A. & Bayard, F. (1987) *Cancer Res.* **47**, 1615–1620.
29. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
30. Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) *Science* **211**, 1437–1439.
31. Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981) *J. Biol. Chem.* **256**, 7990–7997.
32. Hunkapiller, M. W. & Hood, L. E. (1983) *Science* **219**, 650–659.
33. Gill, G. N. & Lazar, C. S. (1981) *Nature (London)* **293**, 305–307.
34. Barnes, D. W. (1982) *J. Cell. Biol.* **93**, 1–4.
35. Schonbrunn, A., Krasnoff, M., Westendorf, J. M. & Tashijian, A. H., Jr. (1980) *J. Cell. Biol.* **85**, 786–797.
36. Imai, Y., Leung, C. K. H., Friesen, H. G. & Shiu, R. P. C. (1982) *Cancer Res.* **42**, 4394–4398.
37. Roberts, A. B., Anzano, M. A., Lamb, L. C., Smith, J. M. & Sporn, M. B. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5339–5343.